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Targeted expression of autoactivated rat Stromelysin-1 in mouse mammary glands was shown to induce hyperplasias and breast cancer, demonstrating that this matrix metalloprotease can act as a natural tumor promoter (Sternlicht *et al.*, 1999). To understand how MMP-3 can influence cancer susceptibility, the yeast two-hybrid system was used to identify interacting proteins as potential novel substrates. The hemopexin domain of MMP-3 interacted with the C-termini of Wnt5a and b in a two-hybrid assay. This interaction is quite specific, since MMP-3 did not interact with the C-termini of Wnt2, 4, 6, 7b or 10b. Furthermore, the hemopexin domains of MMP-2 and MMP-14 did not interact with any of the Wnts tested here. Recombinant MMP-3 catalytic domain can cleave both soluble Wnt5b as well as cell bound Wnt5a and b immunoprecipitated from RIPA cell lysates. Both Wnts were cleaved within the minimal MMP-3 binding domain, consistent with the observed Wnt/catalytic domain two-hybrid interactions. Cleavage of soluble Wnt5a was not observed and may require the presence of a hemopexin domain on MMP-3. These observations suggest that both Wnt5a and b are MMP-3 substrates, but only Wnt5b is co-expressed with MMP-3 in the ductal microenvironment during mammary gland development.

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Table of Contents

Introduction

Matrix metalloproteinases (MMPs) are a family of zinc-dependent extracellular endopeptidases that have been implicated in both physiological and pathological processes. They have traditionally been viewed as effectors of late stages of cancer evolution, as they are positive regulators of angiogenesis and facilitate invasion and metastasis by degrading structural extracellular matrix (ECM) components. However, targeted expression of autoactivated rat Stromelysin-1/MMP-3 in mouse mammary glands was shown to induce hyperplasias and breast cancer, demonstrating that this MMP can act as a natural tumor promoter (Sternlicht *et al.*, 1999). The mechanisms underlying the ability of MMP-3 to modify cell behavior remain poorly understood, as the key *in vivo* substrates for this MMP are still unknown. To understand how MMP-3 can influence cancer susceptibility, the yeast two-hybrid system was used to identify interacting proteins as potential novel substrates. Here I report my findings for the second year of this postdoctoral traineeship award.

Body

Task 1a, 1b and 1c: completed as described in the first annual progress report.

Task 1d: completed. The hemopexin domain of mouse MMP-3 was used to screen a mouse mammary gland GAL4-AD cDNA library (ATCC # 87294) for interacting proteins. Approximately $7.4x10^5$ transformants were tested and 120 candidates were found to activate both reporters (1 out of ± 6200 clones). After demonstration of plasmid dependent activation of both reporters, 98 'true' candidates remained. PCR and restriction analysis indicated that the responsible plasmids contained 54 different cDNA inserts, which were further subdivided in three categories after sequence analysis: a) sequence undetermined (2), b) out of frame cDNA fusions (21) and c) in frame cDNA fusions (31). Table 1 lists the proper cDNA fusions that interact with the mouse MMP-3 hemopexin domain in a two-hybrid assay. The vast majority of interacting clones encoded intracellular proteins, some of which were previously identified in the screen with full-length MMP-3 as 'bait' (mitochondrial serine protease $HTRA_2$, Endo-oligopeptidase A and Leucineaminopeptidase; see alsoTable 3 of my first annual progress report). The interacting proteins of unknown location did not contain a signal peptide or transmembrane domain, suggesting that these molecules are unlikely to be secreted or present at the cell surface. Only two extracellular interacting proteins were identified: 1) One clone contained the last 280 amino acids of ADAMTS-18 (a disintegrin and metalloprotease with thrombospondin motifs). Although nothing is known about this particular multidomain metalloprotease, a recent study on ADAMTS-4 suggests that the C-terminal spacer domain may be required for both binding to the extracellular matrix as well as for substrate specificity (Kashiwagi *et al.*, 2004). ADAMTS-18 contains a furin cleavage consensus site (RX(K/R)R) and is likely activated by this convertase, as it traverses the secretory pathway. Thus this enzyme might not simply be a MMP-3 substrate, but could possibly also act as a MMP-3 activating protease. Since the normal physiological role of this reprolysin family member remains unclear as its potential for dysregulation during malignancy, I decided not to pursue ADAMTS-18 as a potential

MMP-3 substrate. 2) Another clone contained the last 147 amino acids of Wnt5b. Wnt genes encode a large family of secreted protein growth factors that are highly conserved between vertebrate species. Thus far, 19 mouse Wnts have been identified, sharing 27%-83% identity at the amino acid level and a conserved pattern of 23 or 24 cysteine residues (Miller, 2002). Wnts have diverse roles in governing cell fate, proliferation, migration, polarity and death during development. In adults, Wnts function in homeostasis and inappropriate activation of the Wnt signaling pathway(s) has been implicated in a variety of cancers, including breast cancer (Giles *et al.*, 2003). Wnt proteolysis by MMPs would constitute a novel way of modulating the activity of these growth factors and although the role of Wnt5b during mammary gland development is still unknown, cleavage of this molecule has at least the potential to affect cell behavior and could influence tumorigenesis. I therefore have decided to pursue Wnt5b as a potential MMP-3 substrate.

Problems encountered: My two-hybrid screens with the different human and mouse MMP-3 domains revealed that the two mammary gland Gal4-AD cDNA libraries from ATCC are not optimal for identifying potential MMP substrates; they appear to have a poor representation of cell surface and secreted proteins and contain many clones that specifically bind MMP domains through non-relevant two amino acid repeats (see my first annual progress report). It would take a considerable amount of time to generate a new two-hybrid library and since I identified a good candidate substrate for MMP-3, I have decided to postpone the two-hybrid screening with MMP-14 domains for now.

Task 1e: in progress. I have taken two different subcloning approaches in order to identify the domains responsible for the MMP-3/Wnt5b interaction. A deletional approach was used to identify the minimal MMP-3 binding domain within the original Wnt5b two-hybrid clone. It was found that a small stretch of 67 amino acids (residues 258 to 325) mediates binding of Wnt5b to the MMP-3 hemopexin domain in a yeast two-hybrid assay. I am currently in the process of testing an even smaller domain (residues 258 to 313), which is flanked by cysteine residues and is one of the least conserved domains among Wnt molecules. A domain swapping approach was used to identify the Wnt5b binding site in the hemopexin domain of MMP-3. X-ray crystallography has revealed that the hemopexin domain of MMPs is organized as a β-propeller structure with four β-sheets/blades (Woesner and Nagase, 2000). Although the hemopexin domain of Stromelysin-2/MMP-10 is 67% identical to the hemopexin domain of MMP-3, it does not interact with the Wnt5b clone in a two-hybrid assay. Since the four blades of each hemopexin domain are identical in size, I swapped each individual blade from MMP-3 with the corresponding one from MMP-10 to identify the blade(s) involved in binding Wnt5b. This required some extensive site-directed mutagenesis and I am currently in the process of subcloning the different mutant hemopexin domains into 'bait' vector pEG 202 NLS to test them for interaction with Wnt5b.

To further explore the specificity of the MMP-3/Wnt5b interaction, several other domains of these molecules were tested for interaction in the two-hybrid assay. The hemopexin domain failed to bind full length Wnt5b. The catalytic domain interacted with both full length Wnt5b as well as with the C-terminus, while full length MMP-3 failed to bind either. The phenomenon of the two-hybrid assay working better with protein

domains rather than full length molecules is a well known shortcoming of this technique (Bartel and Fields, 1995). I also tested whether the hemopexin domain of MMP-3 could bind the C-terminus of Wnt2, 4, 5a, 6, 7b and 10b, all known to be expressed in the mammary gland (Gavin and McMahon, 1992; Weber-Hall *et al.*, 1994). The C-terminus of Wnt5a is 84% identical to Wnt5b and was the only other Wnt that interacted with MMP-3 in a two-hybrid assay. In addition, the hemopexin domains of MMP-2 and MMP-14, both known to be expressed in the mammary gland (Wiseman *et al.*, 2003), did not interact with any of the Wnts tested here. These observations indicate that not all MMPs can bind all Wnts, which supports the notion that the interaction between MMP-3 and Wnt5a and b is quite specific.

Task 1f: in progress. I originally proposed to further confirm the MMP-protein interaction in yeast by performing cross-linking and co-immunoprecipitation studies. However, I have come to the realization that these experiments do not add to the *in vivo* relevance of this interaction, as both molecules are artificially expressed in the cytoplasm of a non-mammalian cell. It will be more pertinent to co-immunoprecipitate Wnt5a and b with MMP-3 and vice versa from a mammalian cell culture supernatant (see also task 2a). Thus these experiments would be uninformative and have therefore been abandoned. I am in the process of performing β-galactosidase assays to quantitate the strength of all the different two-hybrid interactions described here.

Task 2a: in progress. Double-tagged Wnts were made in order to try demonstrate cleavage of Wnt5a and b by MMP-3 *in vitro*, as they should enable visualization of cleavage even if proteolysis were to occur at one end of the molecule. A myc tag (EQKLISEEDL) was inserted immediately after the predicted signal sequence (between aa residues 42 and 43 of Wnt5a and between aa residues 43 and 44 of Wnt5b) and an HA tag (YPYDVPDYA) was inserted right before the stop codon. These double-tagged proteins are secreted, suggesting that the myc tag does not interfere with cleavage of the signal sequence. However, it is currently unclear whether these molecules are fully functional. The Moloney based pLNCX2 retroviral backbone from Clontech was modified to allow simultaneous expression of both MMP-3 and double-tagged Wnt5a/b from the viral 5'LTR.

Problems encountered: 1) Infection of HEK 293 and NIH 3T3 cells with these viruses was very efficient, but failed to demonstrate cleavage of Wnt5a or b. This was thought to be due to an inability to activate the pro-form of MMP-3. To overcome this problem, the furin cleavage site GLSARNRQKR was inserted between the pro- and catalytic domain of MMP-3 (Pei and Weiss, 1995). Approximately 50% of all MMP-3 was now converted into the 'mature'/active form, as judged by casein zymography. However, cleavage of Wnt5a or b was still not observed. 2) Reverse gelatin zymography revealed that HEK 293 cells secrete three different TIMPs (tissue inhibitors of metalloproteinases), while NIH 3T3 cells secrete high levels of TIMP2. These TIMPs likely inhibit the 'mature'/active form of MMP-3, as concentrated culture supernatant from these cells did not contain proteolytic activity towards a MMP-3 specific fluorescent substrate. Four small interfering double stranded RNAs (siRNAs) were designed and stably introduced in NIH 3T3 cells by retroviral delivery in an attempt to silence TIMP2. Two candidates were

found to knock down TIMP2 at both the mRNA (± 60%) and protein (±90%) level. These cells are currently used for both cleavage and co-immunoprecipitation studies.

Task 2b: in progress. Over a decade of Wnt research has shown that working with Wnts as biological agents is problematic (Cadigan and Nusse, 1997). Unpublished attempts to produce Wnt proteins in *E.coli* and yeast have not worked, nor have baculovirus overexpression systems. Overexpression of Wnt genes in mammalian cultured cells yields very little soluble Wnt in the media, as misfolded Wnt accumulates in the ER while correctly folded Wnt is retained at the cell surface. However, Willert *et al.* (2003) succeeded in purifying soluble Wnt3a by using very large culture volumes in combination with a three step purification method. I would like to avoid wasting a lot of time optimizing the expression and purification of Wnt5a and b. Cell lines stably expressing and secreting double tagged Wnt5a and b have been generated. The culture supernatant of these cells will be used as source of soluble Wnt5a and b to demonstrate cleavage by recombinant MMP-3. The cleavage products can be purified by immunoaffinity chromatography using agarose immobilized anti-myc and anti-HA polyclonal antibodies to identify the cleavage sites by electron spray mass spectroscopy. Purified Wnt5a and b is also not required for generating monoclonal antibodies against the full length and processed forms of these proteins. These can be obtained by screening a human ScFv (single chain antibody fragment) phage display library with small antigenic peptides specific for either Wnt5a or b (Pavlik *et al.*, 2003). Our lab will soon have access to such a library and screening will be initiated when the cleavage sites are known. To establish substrate specificity, tagged Wnt2, 4, 5a, 5b, 6, 7b and 10b will be co-expressed with MMP-2, MMP-3 and MMP-14 in NIH 3T3 cells knocked down for TIMP2. To this extent a furin cleavage site was introduced in MMP-2, while MMP-14 is normally processed by this convertase.

Problems encountered: 1) The catalytic domain of MMP-3 and the full length enzyme were expressed in *E. coli*, to obtain large quantities of recombinant MMP-3 free of other metalloproteases. Both proteins were expressed at decent levels (± 20 mg/l of *E. coli* culture), but accumulated in inclusion bodies. The insoluble proteins were solubilized in 8M urea and refolded by dropwise dilution. Unfortunately, the full length enzyme became partially activated during this process and cleaved of its hemopexin domain. I am currently in the process of refolding these proteins by dropwise dilution in the presence of the general MMP inhibitor 1,10-phenantroline to prevent auto-degradation. 2) The recombinant catalytic domain of MMP-3 can cleave cell bound Wnt5a and b immunoprecipitated from RIPA cell lysates. However, it appears to only cleave soluble Wnt5b. Cleavage seems to occur within the minimal MMP-3 interacting domain as judged by mobility shift on SDS-PAGE and can be blocked by the MMP inhibitors GM-6001 and EDTA. It will be necessary to confirm these observations with full length enzyme, especially since the hemopexin domain is involved in binding the Wnt proteins.

Task 2c: in progress. Weber-Hall *et al.* (1994) determined by in situ hybridization that Wnt5a expression is stromal throughout ductal development and pregnancy. The same authors found that Wnt5b expression is ductal + lobular and is first observed in 12 week old virgins. A more global gene expression analysis has recently been performed by

Hosein Kouros-Mehr, a talented MD/PhD student in our lab who is interested in identifying transcripts that are specifically upregulated in the different structures of the mammary gland. He dissected out terminal end buds (TEBs) and ducts from mammary glands of five week old virgin mice and compared their transcriptional profile with that from distant stroma using microarray technology. He found that Wnt5b and MMP-3 expression was restricted to the ductal microenvironment, while the Wnt5a transcript was only present in the microenvironment of TEBs. Although these observations need to be independently confirmed by immunohistochemistry, they suggest that Wnt5b could be a genuine MMP-3 substrate *in vivo*, as both genes are expressed at the same time and in the same compartment of the mammary gland.

Problems encountered: 1) A lack of commercially available antibodies that specifically recognize Wnt5a, Wnt5b and mouse MMP-3 has prevented me from trying to *i*) detect processed Wnts in the mammary gland and *ii*) co-immunoprecipitate Wnt5a and/or b with MMP-3 from mammary gland lysates. An anti-Wnt5a goat polyclonal antibody is available from NEUROMICS, but recognizes several other Wnts including Wnt5b. Anti-mouse MMP-3 antibodies are not available and antibodies raised against human MMP-3 do not seem to cross-react with their mouse homolog, despite the fact that these molecules are 83% identical. 2) Our transgenic mouse line overproducing the autoactivating rat Stromelysin-1 transgene from the murine WAP promotor was terminated due to a loss of transgene expression. However, a collegue is in the process of making a similar mouse line by introducing the same transgene behind a tet-regulatable promotor.

Task 3: not yet initiated.

Key Research Accomplishments

- The hemopexin domain of MMP-3 can interact with the C-terminal portion of Wnt5a and b in a yeast two hybrid assay
- The catalytic domain of MMP-3 can cleave *i*) cell bound Wnt5a and b immunoprecipated from RIPA cell lysates and *ii*) soluble Wnt5b, but not soluble Wnt5a
- MMP-3 and Wnt5b are both expressed in the ductal microenvironment during mammary gland development

Reportable Outcomes

None to date

Conclusions

Although the identification of MMP interacting proteins as potential substrates by yeast two-hybrid screening was less successful than anticipated, I still believe that this is

a worthwhile technique as it is unlikely that I would have isolated Wnt5b by a proteomic approach. However, only the hemopexin domain should be used as 'bait', since the catalytic domain is 'sticky' and the full length MMPs are poorly folded in the yeast cytoplasm. To further increase the success rate of this method, one should enrich for clones encoding membrane and secreted proteins by using rough-ER bound mRNA for the construction of the two-hybrid library (Diehn *et al.*, 2000).

The hemopexin domain of MMP-3 interacts with the C-termini of Wnt5a and b in a two-hybrid assay. This interaction seems to be quite specific, as MMP-3 does not interact with the C-termini of Wnt2, 4, 6, 7b and 10b. In addition, the hemopexin domains of MMP-2 and MMP-14 do also not interact with any of the Wnts tested here. Recombinant MMP-3 catalytic domain can cleave both soluble Wnt5b as well as cell bound Wnt5a and b immunoprecipated from RIPA cell lysates. Both Wnts were cleaved within the minimal MMP-3 binding domain, consistent with the Wnt/catalytic domain two-hybrid interactions. Cleavage of soluble Wnt5a was not observed and may require the presence of a hemopexin domain on MMP-3. These observations suggest that both Wnt5a and b are MMP-3 substrates, but only Wnt5b is co-expressed with MMP-3 in the ductal microenvironment during mammary gland development.

The role of Wnt5a and b in the mammary gland is not well understood. Wnt5a is the best-studied molecule of the two and its expression has been analyzed in human breast cancers, where it was found to be up-regulated in both benign proliferations and invasive cancers (Lejeune *et al.*, 1995). Targeted gene disruption in mice has thus far not revealed a mammary gland phenotype, as Wnt5a $^{-/-}$ animals die shortly after birth (Yamaguchi *et al.*, 1999). It will be interesting to see whether inappropriate proteolysis of these molecules by MMP-3 contributes to breast cancer induction and/or progression.

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Table 1. Mouse MMP-3 hemopexin domain interacting proteins

	Total number identified	Number of independent clones
I Intracellular proteins		
1) TAX 1 binding protein 1 homolog	14	5
2) Endooligopeptidase A	7	3
3) 26S proteasome subunit 3	4	2
4) Mitochondrial serine protease $HTRA_2$	3	2
5) Zip kinase	3	1
6) Thioredoxin 1	2	2
7) Cyclin M_3	2	1
8) Cytokeratin 19	1	1
9) Gluthatione-S-Transferase	1	1
10) Grpe-like 2	1	1
11) Nibrin	1	1
12) Leucineaminopeptidase 3	1	1
II Proteins of unknown location		
1) Hypothetical protein B230208H176	6	2
2) Hypothetical protein D7ERTD671E	3	1
3) Eso 3	2	1
4) Ganglioside expression factor 2	2	1
5) LAG protein / NMDA receptor glutamate binding chain	1	1
6) Hypothetical protein MGC6696	1	1
7) Riken cDNA C030006K11	1	1
III Extracellular proteins		
1) AdamTS-18 / Reprolysin	1	1
2) Wnt-5B	1	1